ATTACHMENT II – PROTOCOL

ECOLAB
Study Identification Number 1200053

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: Aqualogic Non-Food Contact Surface Sanitizing Efficacy 30 seconds Exposure Time

EPA REG. NO.: 1677-

STUDY IDENTIFICATION NUMBER: 1200053

PROPOSED STUDY INITIATION/COMPLETION DATES

Initiation May 18, 2012

Completion July 18, 2012

DESCRIPTION OF STUDY OBJECTIVE

Aqualogic (EPA Registration No. 1677-) will be tested according to Ecolab Microbiological Services SOP Method MS016-23; *Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)* to determine non-food contact surface sanitizing efficacy against *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048 after a 30 seconds exposure time at room temperature (15-30°C) when diluted to 0.0660% free available chlorine in sterile Milli-Q or laboratory purified water per the Confidential Statement of Formula (CSF). The actual dilutions which will be performed for the test substance use-solutions will be determined subsequent to the chemical quality verification to deliver the required level of active ingredient, and documented in the raw data. The test substance will be challenged by the addition of 5% fetal bovine serum to the test systems. ASTM Method E1153-03 (reapproved 2010) was the test method utilized in making the sanitizing claim.

TEST SUBSTANCE IDENTIFICATION

Test Substance Name: Aqualogic

Batch Identification:

1. **051512DT**
2.
3. **050112DT***

*Indicates the batch that will be tested as the 60 day aged batch.

One of the test substance batches will be amended into the study. All three batches will be used to determine the use-solution chemical quality verification analysis.

An aliquot of each test substance batch will be retained in the retention cabinet at ECOLAB Schuman Campus until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in ECOLAB Microbiological Services laboratory until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, chemical quality verification in-life inspection, chemical quality verification in-life data audit, pesticide efficacy in-life and final report are proposed to be inspected by the ECOLAB Quality Assurance Unit (QAU) in accordance with their current Standard Operating Procedures. The following proposed ECOLAB QA inspections are for planning purposes only and may change. ECOLAB QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in ECOLAB QA inspections from those proposed below will not require revision of this protocol.

A. Proposed QAU Monitoring

Protocol Audit
Chemical Quality Verification In-Life Inspection
Chemical Quality Verification Data Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

CHEMICAL QUALITY VERIFICATION

A. Proposed Experimental Initiation/Termination Dates

Experimental Initiation Date: May 24, 2012

Experimental Termination Date: July 1, 2012

B. Method

Chemical analysis will be performed on each test substance batch to determine the concentration of the active ingredient under ECOLAB GLP study number 1200052. Chemical analysis will also be performed on the test substance use-solution. The use-solution preparation will be documented in the raw data.

The test substance is a ready-to-use product that will be diluted at or below the lower certified limit for the active ingredient. The following calculation will used to determine the amount of test substance in a 200 g use-solution diluted to 660 ppm (or 0.0660%) free available chlorine:

ppm at LCL = (% LCL/100) (specific gravity) 10^6 =
ppm at LCL = (0.0660%/100) X (0.999) X 10^6 = 659 ppm

Amount of Test Substance needed to be at or below the LCL =

$$\frac{\text{ppm at LCL} \times 100 \times \text{g amount of use-solution to be made}}{(\%\ \text{active})\ 10^6} = \text{grams of Test Substance}$$

In order to prepare the use-solution using weight to weight measurements, the specific gravity was incorporated into the calculations resulting in 659 ppm (or 0.0659%) free available chlorine as the lower certified limit.

The chemical quality verification will be performed by the Analytical Lab using the method listed below. The method has been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution samples.

QATM-007; Available Chlorine
Available chlorine content is determined by reduction of the chlorine to chloride by iodide ion. The iodine liberated by this reaction is then determined by titration with sodium thiosulfate, either manually or potentiometrically with an automatic titrator.

The most current QATM will be used during the course of this study for the chemical and physical analysis.

C. Interpretation of Results

The concentration of the active ingredient in the test substance batches will be judged acceptable for pesticide efficacy testing if within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Free Available Chlorine*	0.0660%	0.1030%

*The equivalent weight of NaOCL (sodium hypochlorite) to the equivalent weight of Cl_2 (Chlorine) is 37.2/35.5 = 1.05. Dividing the sodium hypochlorite concentration by the ratio of the equivalent weight of sodium hypochlorite to the equivalent weight of chlorine results in the free available chlorine concentration.

The concentration of the active ingredients in the test substance diluted to the lower certified limit (test substance use-solution) will be judged acceptable for pesticide efficacy testing if within the acceptance limit of 0.0594 – 0.0726% available chlorine.

After diluting the ready-to-use test substance to the lower certified limit of 0.0660% free available chlorine, the nominal concentration of the active ingredient is <1.0%. Therefore, the Calculated Lower Acceptance Limit and Calculated Upper Acceptance Limit for available chlorine will be expanded to accommodate method variability and suitable rationale. The expanded ranges are based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

Calculated Lower Acceptance Limit for available chlorine
= [0.0660% - (0.0660 x 0.1)] = 0.0594%
Calculated Upper Acceptance Limit for available chlorine
= [0.0660% + (0.0660 x 0.1)] = 0.0726%

The chemical quality verification raw data will be reported in the final report of this study.

PESTICIDE EFFICACY TESTING

A. <u>Proposed</u> Experimental Start/Termination Dates

Experimental Start	May 24, 2012
Experimental Termination	July 1, 2012

B. Methods

Pesticide efficacy data will be generated by the Microbiology Lab using the most current methods listed below. See the specific methods in the Protocol Appendix.

Method Number	Method Name
MS002-15	*Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims*
MS016-23*	*Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)*
MS088-17	*Test Substance Use-Solution Preparation for Analysis*

*MS016 will be followed with the following exceptions:
The test system culture used for the efficacy test will be a 48 ± 4 hour culture. 0.02 mL of test system will be inoculated onto each carrier. The carriers will be dried at 35 ± 2°C in a constant humidity desiccator for 35 minutes. The control carriers will be treated with sterile lab purified (or Milli-Q) water.

Test Method Requirement and Test System Justification

The following apply when determining the effectiveness of a hospital disinfectant; 60 carriers are required on each of three samples, representing different batches one of which is greater than 60 days old. The required organisms are *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048. ASTM Method E1153-03 (reapproved 2010), Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces, for the above stated organisms are recommended based on the U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2300 Sanitizers for Use on Hard Surfaces –Efficacy Data Recommendations March 12, 2012. Also, U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2000 General considerations for Public Health Uses of Antimicrobial Agents March 12, 2012 applies to this study.

Test Method Justification

Ecolab Microbiological Services SOP Method MS016-23; *Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)* will be the test method utilized in this study.

Test Systems and Identification

The test systems which will be utilized for this procedure *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048. Identification will be performed by observing the colony morphology.

Organic Soil Load

5% Fetal Bovine Serum

Test Substance Diluent

Sterile Milli-Q water or Sterile laboratory purified water

Test Substance Concentration

Antimicrobial efficacy testing will be performed with **Aqualogic** diluted to at or below the lower limit of 660 ppm.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Available Chlorine	0.0660%	0.1030%

The dilution procedure is based on results of the Chemical Quality Verification study. To achieve dilution of the ready-to-use test substance to at or below the lower certified limit of available chlorine, the test substance batches will be prepared based on the available chlorine results and documented in the raw data. The following calculation will be used to determine the dilution procedure for each test substance batch to result in the lower certified limit of available chlorine.

ppm at LCL = (% LCL/100) (specific gravity) 10^6 =
ppm at LCL = (0.0660%/100) X (0.999) X 10^6 = 659 ppm

Amount of Test Substance needed to be at or below the LCL =

$$\frac{\text{ppm at LCL} \times 100 \times \text{g of use-solution to be made}}{(\%\ \text{active})\ 10^6} = \text{grams of Test Substance}$$

Test Surface

Stainless Steel, 25 x 25 mm (1 x 1")

Exposure Time/Temperature

The test systems will be exposed to the test substance for 30 seconds at room temperature (15-30°C).

Neutralizer/Subculture Medium

0.5% Sodium Thiosulfate

Plating Medium

Tryptone Glucose Extract Agar

Incubation Time/Temperature

S. aureus ATCC 6538 plates will be incubated for 48 ± 4 hours at 35 ± 2°C.
E. aerogenes ATCC 13048 plates will be incubated for 48 ± 4 hours at 30 ± 2°C.

Test Controls

The following controls will be incorporated with the test procedure for each test system:

a. Inoculum Count
b. Inoculum Numbers Control Squares
c. Neutralization Controls
d. Test Substance Diluent Sterility Control
e. Organic Soil Sterility Control
f. Test System Purity

Details on each of the above controls can be found in Ecolab SOP MS016-23 located in Protocol Appendix.

Interpretation of Test Results

The performance standard for a non-food contact sanitizer is at least a 99.9% log reduction in the numbers of both *S. aureus* ATCC 6538 and *E. aerogenes* ATCC 13048.

DATA RETENTION

Following completion of the study, an exact copy of the final report and the original raw data and protocol will be transferred to ECOLAB Archives at the ECOLAB Schuman Campus in Eagan, MN or an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the retention cabinet at the ECOLAB Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

- **Name and Address of Sponsor**

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Sponsor — Date: 05/18/2012

Study Director — Date: 18 May 2012

PROTOCOL APPENDIX

Microbiological Services (MS) Methods:

MS002-15	*Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims*	Pages 1-3
MS016-23	*Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)*	Pages 1-9
MS088-17	*Test Substance Use-Solution Preparation For Analysis*	Pages 1-6

ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: **Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims**

NUMBER: **MS002-15**

EFFECTIVE: **04/01/11**

1.0 PURPOSE

The addition of organic soil to a test procedure is necessary to allow for the one-step cleaner-disinfectant or cleaner-sanitizer claim.

2.0 SCOPE

2.1 An antimicrobial product that bears the label claim of a one-step cleaner-disinfectant or cleaner-sanitizer, or one intended to be effective in the presence of organic soil, must be tested for efficacy by the appropriate method(s) which have been modified to include a representative soil such as 5% fetal bovine serum (e.g. blood serum).

3.0 STORAGE & HANDLING INSTRUCTIONS FOR FETAL BOVINE SERUM

3.1 Fetal bovine serum is to be stored at ≤ -10°C and used prior to the expiration date on the bottle label.

3.2 Fetal bovine serum may be thawed and dispensed into vials. Thawed fetal bovine serum may be stored at 2 - 8°C for up to two months from the date thawed.

3.3 Document the following information on Form 3070:

- Fetal Bovine Serum manufacturer
- Lot number
- Bottle identification
- Bottle expiration date
- Thawed/dispensed date
- New expiration date
- Initial & Date

TITLE: **Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims**

NUMBER: **MS002-15**

4.0 TEST REQUIREMENTS

4.1 A suggested procedure to simulate in-use conditions where the antimicrobial agent is intended to treat dry inanimate surfaces with an organic soil load involves contamination of the appropriate carrier surface with each test microorganism culture containing 5% (v/v) fetal bovine serum (e.g. 19 mL test system and 1 mL fetal bovine serum) prior to the specified carrier-drying step in the method.

Note: The organic soil should be added to the test system suspension prior to inoculation of the test surface or test substance.

4.2 A 5% level of organic soil is considered appropriate for simulating lightly or moderately soiled surfaces. When the surface to be treated is heavily soiled, a cleaning step must be recommended prior to application of the antimicrobial. The effectiveness of antimicrobial agents must be demonstrated in the presence of a specific organic soil at an appropriate concentration level according to a specific label claim.

4.3 If an antimicrobial product has a soap scum claim, an appropriate method of simulation would be to add a 0.005% concentration of sodium stearate. A 1:20 dilution of a 0.1% solution of sodium stearate is made into the culture inoculum for a final concentration of 0.005%

4.4 An alternate method of introducing the organic soil where the antimicrobial agent is not tested against a dry inanimate surface involves adding 5% organic soil to the use-solution of a product prior to inoculation with the test system (e.g. 4.75 mL use-solution + 0.25 mL fetal bovine serum before adding 0.5 mL of the required level {5 x 10^6/mL} of conidia).

5.0 REGULATORY EFFICACY REPORTS

5.1 The level and type of organic soil must be stated in the protocol, raw data and the final efficacy report. The method of incorporation of organic soil must also be stated (e.g. 5% fetal bovine serum added to the test system suspension for the inoculation of the test surface or test substance).

5.2 The Certificate of Analysis for the fetal bovine serum shall be included in the study file.

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Standard Operating Procedure

Ecolab Controlled Document

TITLE: Organic/Inorganic Soil Addition for One-Step Cleaner Disinfectant or Sanitizer Claims

NUMBER: MS002-15

6.0 RECORD MAINTENANCE

6.1 Records will be stored in the Test System binder located in Microbiological Services. Records from the current and previous year will be kept in the Microbiological Services Equipment Maintenance binder. All earlier records will be archived in the first quarter of the current year. For example, records from 2010 will be archived by March of 2012. Records will be transferred to ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

7.0 RELATED FORMS

7.1 Form 3070: Fetal Bovine Serum

8.0 REFERENCES

8.1 EPA DIS/TSS-2, 25 Jan. 79 (page 2 of 3)

9.0 MOST RECENT REVISION SUMMARY

Revised 3.2.

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Prepared by: Lavinda Hilen Date: 3/14/11

Quality Assurance: Sherri St Clair Date: 14 Mar 2011

Management: [signature] Date: 14 March 2011

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ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: **Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)**

NUMBER: **MS016-23**

EFFECTIVE: **12/01/11**

1.0 PURPOSE

To evaluate the antimicrobial efficacy of sanitizers on pre-cleaned inanimate, nonporous, non-food contact surfaces. It may also be used to evaluate the antimicrobial efficacy of one step cleaner/sanitizer formulations recommended for use on lightly soiled, inanimate, nonporous, non-food contact surfaces.

2.0 CULTURE MEDIA

2.1 AOAC Nutrient Broth
2.2 Nutrient Agar
2.3 Tryptone Glucose Extract Agar
2.4 Other culture media as appropriate for the test system

3.0 NEUTRALIZER BROTH

3.1 Letheen broth
3.2 Other neutralizer media appropriate for the test material

4.0 APPARATUS

4.1 Glassware

4.1.1 Disposable glass or plastic pipets, volumetric flasks, Pyrex beakers, 20 x 150 mm or 25 × 150 mm Pyrex test tubes and 2 oz. medicine jars. Autoclave to sterilize. Plastic 2 oz. medicine jars may also be used.

4.2 Test Tube Racks

4.2.1 Any convenient style capable of holding 20 x 150 mm or 25 × 150 mm test tubes

4.3 Petri Dishes

4.3.1 Sterile disposable 15 × 100 mm petri dishes

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TITLE: Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)

NUMBER: MS016-23

4.4 Thermocouple

4.4.1 Capable of monitoring test temperature throughout the test. Record this data with raw data.

4.5 Test Squares

4.5.1 The size of the test squares should be 1" × 1" or other appropriate size.

4.5.2 The test square should represent the type of surface(s) recommended for treatment in the directions for use such as glass, metal, unglazed or glazed ceramic tile, or porcelain, etc. The type of surface must be documented.

4.6 Timer

4.6.1 A reliable stopwatch or lab timer capable of measuring elapsed time in seconds and minutes.

4.7 Incubators

4.7.1 Capable of providing temperature chosen in the range 30 - 35°C, ± 2°C.

4.8 Orbital/Rotary Shaker
4.9 Large Desiccator
4.10 Vortex Mixer

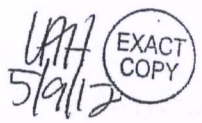
5.0 REAGENTS

5.1 Phosphate Buffer Dilution Water (PBDW)
5.2 0.01% isooctylphenoxypolyethoxyethanol (Triton X-100)
5.3 Blood Serum and/or other appropriate organic load – if required. (see MS002)
5.4 Diluent (refer to MS008 if preparing synthetic hard water)
5.5 Glycerin solution, 86.5% in distilled/deionized water

6.0 TEST REQUIREMENTS

6.1 Each test substance must be tested against each of the following test systems:

- *Staphylococcus aureus* ATCC 6538 and
- *Klebsiella pneumoniae* ATCC 4352 OR
- *Enterobacter aerogenes* ATCC 13048 or 15038



Other test systems may be used, utilizing appropriate medium and incubation conditions.

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6.2 If the product is to be a one-step cleaner-sanitizer, an appropriate organic soil load such as 5% blood serum must be added to the test system inoculum.

7.0 TEST SYSTEM PREPARATION

- *Staphylococcus aureus* ATCC 6538 and
- *Klebsiella pneumoniae* ATCC 4352 or
- *Enterobacter aerogenes* ATCC 13048 or 15038

7.1 A minimum of three consecutive transfers but less than 15 total transfers in AOAC Nutrient Broth need to be made before using to inoculate for testing.

7.2 If only one transfer is missed per seven day period, it is not necessary to repeat the three consecutive transfers.

7.3 If two or more transfers are missed, repeat with the three consecutive transfers.

7.4 Transfers are made on a 24 hour schedule. The last consecutive transfer used to inoculate for the test should be a 24 ± 4 hour test system.

7.5 Inoculate a sufficient number of culture media broth tubes for the test. Incubate for 24 ± 4 hours at temperature appropriate for the test organism.

7.6 Vortex the culture to mix. Allow culture to stand for at least 15 minutes; decant upper two thirds of suspension for use in test.

7.7 Add soil challenge (blood serum or other appropriate organic soil) to the culture if required by a test protocol.

8.0 TEST SUBSTANCE PREPARATION

8.1 If dilution of the test substance is required, use greater than 1.0 mL or 1.0 g of product.

8.2 The use solution must be tested within three hours of preparation or within the known stability of the solution.

9.0 OPERATING TECHNIQUE

9.1 Inoculation of Test Squares

9.1.1 Place sterile squares in a sterile, plastic petri dish. Make sure the test squares don't touch each other.

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9.1.2 Inoculate each sterile square with 0.01 to 0.03 mL of a 24 ± 4 hour culture of the test system. Spread the inoculum to within 1/8" of the edges of the square.

9.1.3 Inoculate five sterile test squares for each test system.

9.1.4 Place petri plates containing the inoculated squares in an incubator at desired temperature (30 – 35°C, ± 2°C) for 30 – 40 minutes.

9.1.5 Use of a constant humidity chamber (desiccator) for drying the inoculated test squares may aid in achieving desired control numbers. The desiccator should be prepared at least one day before use in testing by putting about 500 mL of 86.5% glycerin solution into the bottom of the desiccator and holding it (covered) to equilibrate in the incubator that will be used for drying the inoculated test squares.

9.2 Inoculum Count

9.2.1 While the test squares are drying, plate 1.0 mL and 0.1 mL of a 10^{-6} (or other appropriate) dilution of the test system broth in triplicate. Incubate for 48 ± 4 hours at temperature appropriate for the test organism and then count the colonies to determine the number of colony forming units per mL.

9.3 Treatment of Inoculated Test Squares

9.3.1 Transfer the five squares of each test system to five sterile medicine jars using aseptic technique.

9.3.2 At time zero, apply 5 mL of the test substance to inoculated square No. 1. At one minute, apply 5 mL of the test substance to inoculated square No. 2. Treat square No. 3 in a like manner at two minutes, square No. 4 at three minutes and square No. 5 at four minutes.

Note: This protocol is for a five minute exposure time. Shorter exposure times would require different intervals.

9.3.3 At five minutes on timer, transfer 20 mL of neutralizer into jar No. 1 and rotate the jar on an even plane approximately 50 rotations to suspend the surviving organisms in the neutralizer solution. An orbital/rotary shaker may be used. At six minutes, transfer 20 mL of neutralizer into jar No. 2 and agitate as before. Continue addition of neutralizer to jars No. 3, No. 4 and No. 5, in that order, at one minute intervals and agitate.

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9.3.4 Plate 1.0 mL and 0.1 mL of the neutralizer solution from each of the five jars in duplicate using pour plate technique. Incubate at 35 ± 2°C for 48 ± 4 hours. Incubate other organisms as appropriate.

10.0 CONTROLS

10.1 Inoculum Numbers Control Squares

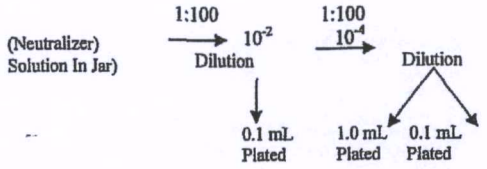
10.1.1 Proceed as in 9.1 inoculating three sterile squares.

10.1.2 Proceed as in 9.3 using one of the following in place of test substance:

10.1.2.1 The test substance not containing the active ingredients
10.1.2.2 Sterile deionized water
10.1.2.3 Sterile deionized water containing 0.01% isooctylphenoxypolyethoxyethanol (Triton X-100)

10.1.3 Five minutes (or other exposure time) after treating control square No. 1, cover it with 20 mL of the neutralizer solution. Rotate the jar vigorously on an even plane approximately 50 rotations to suspend the surviving organisms in the neutralizer solution. An orbital/rotary shaker may be used. In a like manner, add 20 mL of the neutralizer to control squares No. 2 and No. 3 after treating them with 5 mL of 0.01% TritonX-100. Agitate the jars containing these squares as was done for square No. 1.

10.1.4 Plate 0.1 mL of a 10^{-2} dilution of the neutralizer solution from each of the three control jars in duplicate using pour plate technique. Also plate 1.0 mL and 0.1 mL of a 10^{-4} dilution of the neutralizer solution from each of the three control jars in duplicate using pour plate technique. Other appropriate dilutions may be prepared and plated. Incubate the same as the test plates.



10.1.5 Repeat steps 10.1 (1 - 4) using each test system. For each test system, test inoculum numbers controls after testing of test substances.

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10.2 Neutralization Method (created from ASTM E 1054-08)

10.2.1 A neutralization method check should be performed on each test system for each test substance. If more than one use-solution concentration is used, test the most concentrated solution. Testing should be performed as follows:

- Test A = Add 20 mL of neutralizer to 5 mL of the test substance use-solution and mix. Add 0.2 mL of ~10^4 CFU/mL test system suspension and mix.
- Test B = Add 20 mL of neutralizer to 5 mL of the test substance diluent (use sterile Milli-Q water if test substance was not diluted) and mix. Add 0.2 mL of ~10^4 CFU/mL test system suspension and mix.
- Test C = Add 0.2 mL of ~10^4 CFU/mL test system suspension to 25 mL of phosphate buffered dilution water and mix.

10.2.2 Let tests stand for 30 minutes and no greater than two hours, then enumerate by plating 0.1 and 1 mL in duplicate using pour plate technique.

10.2.3 Incubate at 48 ± 4 hours at test system specific temperature.

10.3 Test Substance Diluent Sterility

10.3.1 Plate 1 mL of the test substance diluent used in the test using a suitable culture medium.

10.4 Organic Soil Sterility Control (if applicable)

10.4.1 Plate 1 mL of the organic load used in the test using a suitable culture medium.

10.5 Test System Purity

10.5.1 Inoculate the test system suspension onto Tryptic Soy Agar with 5% sheep's blood (e.g. Blood agar plate) and streak for isolated colonies. If the test system does not grow on Blood Agar, use an alternate agar-based medium that supports its growth.

10.5.2 Following incubation, perform a Gram stain and record colony morphology and Gram stain results.

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11.0 CALCULATIONS

11.1 Determine CFU per Milliliter of Neutralizing Solution

11.1.1 Determine the number of CFU per milliliter of neutralizer solution for both the test squares and the inoculated control squares (add the total number of colony forming units on each of the duplicate countable plates and divide by two to obtain the number of CFU per plate). Multiply the CFU per plate by the dilution factor and divide by the volume plated.

$$\text{Average CFU/mL} = \frac{(\text{Average CFU/Plate}^{a})(\text{Dilution Factor}^{b})}{\text{Volume Plated}}$$

[a] If the average CFU/plate is zero, use < 1 as average CFU/plate.

[b] The dilution factor is the reciprocal of the dilution used (e.g. if 10^{-3} dilution used, dilution factor is 1000). Choose the dilution used for calculation according to colony count rules in MS087.

11.2 Determine Number of CFU per Square

11.2.1 Multiply the number of CFU per milliliter of neutralizer solution by 25 to provide the number of CFU per square.

11.3 Determine Log_{10} CFU/Square

11.3.1 Determine the Log_{10} of each CFU per square.

11.4 Geometric Mean of CFU on Control Squares

11.4.1 Determine the geometric mean of the number of organisms surviving on the three inoculated control squares by the following equation:

$$\text{Geometric Mean} = \text{Antilog}\left[\frac{(\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3)}{3}\right]$$

Where X equals the number of organisms surviving per control square.

11.4.2 An average of at least 7.5×10^5 CFU must be present on the inoculated control squares for the test to be valid.

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TITLE: **Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)**

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11.5 Geometric Mean Number of CFU on Test Squares

11.5.1 Determine the geometric mean of the number of CFU on the five test squares by the following equation:

$$\text{Geometric Mean} = \text{Antilog}\left[\frac{(\text{Log}_{10}Y_1 + \text{Log}_{10}Y_2 + \text{Log}_{10}Y_3 + \text{Log}_{10}Y_4 + \text{Log}_{10}Y_5)}{5}\right]$$

Where Y equals the number of CFU surviving per test square.

11.6 Percent Reduction

11.6.1 Use the following equation to calculate percent reduction:

$$\%\text{Reduction} = \frac{a-b}{a}(100)$$

a = Geometric mean number of organisms surviving on the inoculated control squares (as determined in 11.4.1)

b = Geometric mean number of organisms surviving on the test squares (as determined in 11.5.1)

Note: If no growth is seen on the 1.0 mL plate, the result is recorded as < 25.

12.0 INTERPRETATION OF RESULTS

12.1 At least a 99.9% reduction in the numbers of both (if using *S. aureus* and *K. pneumoniae* or *E. aerogenes*) test organisms must be obtained.

12.2 The average plate counts for neutralization control tests A and B are within a 0.3 $\log_{10}$ of the average plate counts for the control test C.

13.0 RELATED FORMS

13.1 Form 3013: Non-Food Contact Sanitizer Method

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TITLE: Non-Food Contact Sanitizer Method Sanitizer Test (for inanimate, non-food contact surfaces)

NUMBER: MS016-23

14.0 REFERENCES

14.1 ASTM E 1153-03(2010)el
14.2 DIS/TSS-10, 1976
14.3 ASTM E 1054-08 Neutralization Method
14.4 MS002: Organic Soil Addition for One-Step Cleaner-Disinfectant or Sanitizer Claims
14.5 MS008: Synthetic Hard Water Preparation & Standardization
14.6 MS040: Media Preparation & Storage-Media & Chemicals
14.7 MS087: Colony Count Methods

15.0 MOST RECENT REVISION SUMMARY

Minor format and grammar corrections throughout. Changed the neutralizer validation criterion from 0.5 log to 0.3 log in 12.2.

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Prepared by: Linda Briscoe Date: 11/29/11
Quality Assurance: Merri St. Clair Date: 29 NOV 2011
Management: Mary J Bruce Date: 29 Nov 2011

ECOLAB
MICROBIOLOGICAL SERVICES

5

Ecolab, Inc. Controlled Document — Standard Operating Procedure

TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-17**

EFFECTIVE: **08/01/11**

1.0 PURPOSE

To describe the preparation and active ingredient analysis of a diluted test substance (test substance use-solution). Use-solution analysis is included with pesticide efficacy studies, chemical quality verification studies and contract lab studies to verify that the active ingredient concentration corresponds to the dilution made for the claimed active ingredient concentration in the undiluted test substance.

2.0 PROCEDURE

2.1 Typically, use-solutions are prepared as follows:

2.1.1 Use-solutions prepared according to the label are for chemical quality verification (CQV) studies

2.1.2 Use-solutions prepared at the Lower Certified Limit (LCL) are for efficacy studies

2.1.3 Use-solutions prepared at the Upper Certified Limit (UCL) are for contract lab TOX studies

2.2 Determine the concentration of active ingredient in the test substance concentrate to verify it is within claimed limits. Perform the analysis for each active ingredient in the product.

2.3 Deionized water may be used as the test substance diluent or the test substance diluent (e.g. hard/soft water or label instructed diluent) may be prepared in the same manner as used for pesticide efficacy testing.

2.4 Prepare the test substance use-solution according to label instructions or as specified in protocol using diluent as described in 2.3. This use-solution should be labeled according to M032.

Example: A 1:64 dilution is 1 part test substance, 63 parts diluent.

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2.5 Analyze the test substance for active ingredient concentration using the same validated QATM that is, or will be, included in the finished good Bill of Quality (BOQ).

Note: The method used to measure active ingredient concentration in the use-solution may have limited sensitivity, accuracy and precision for quantitating the minimal levels of active ingredient found in many use-solutions. These factors may need to be considered when interpreting results. Any modifications to the QATM to adjust for this should be specified in the protocol.

2.6 Analyze the results. The active ingredient concentration in the use-solution should correspond to the dilution made for the claimed active ingredient concentration in the concentrate (e.g. EPA Upper & Lower Certified Limits) and to 40 CFR § 158.350 Certified Limits unless otherwise noted in the protocol. A scientific explanation must accompany any result which does not correspond to the dilution made for the claimed active ingredient level in the concentrate.

3.0 Formulas to Determine Use-solution Amounts and Acceptance Criteria

3.1. Dilution Factor (DF) Determination

3.1.1 Dilution Factor by Volume (DF_{vol})

Example: Dilution Factor (DF_{vol}) $= \left(\frac{1\,\text{oz}}{1\,\text{gallon}}\right)\left(\frac{1\,\text{gallon}}{128\,\text{oz}}\right) = 0.0078$

3.1.2 Density/Specific Gravity (SG) Calculation

Obtain density or specific gravity values from confidential statement of formula (CSF) or suitable documentation. Convert as necessary to g/mL or unitless for SG.

Conversion Example: $\left(\frac{9.2\,\text{lbs}}{\text{gallon}}\right)\left(\frac{1\,\text{gallon}}{3785.412\,\text{mL}}\right)\left(\frac{453.5924\,\text{g}}{1\,\text{lb}}\right) = 1.102\,\text{g/mL}$

$$\text{Density of Product} = \frac{\text{mass (g)}}{\text{volume (mL)}};\ \text{Specific Gravity} = \frac{\text{Density of Product}}{\text{Density of Water (1.0 g/mL)}}$$

$$\text{Density of Product} = 9.2\ \text{lbs/gal} \sim 1.102\ \text{g/mL};\ \text{Specific Gravity} = \frac{1.102\ \text{g/mL}}{1.0\ \text{g/mL}} = 1.102$$

3.1.3 $DF = DF_{vol} \times SG$

$DF = 0.0078 \times 1.102 = 0.0086$

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3.2. Use-solution prepared per label (e.g. 1000 g use-solution prepared at 1 oz/gallon dilution)

3.2.1 Target mass (g) of product = [Total use-solution mass (g)] × DF

Target mass (g) of product = 1000 g × 0.0086 = 8.6 g

3.2.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Target mass (g) of diluent = 1000 g – 8.6 g = 991.4 g

3.2.3 Include a range of ± 0.03 g (~ 1 drop) or ± 0.3 g (~ 10 drops) to target masses when preparing use-solutions.

Note: any appropriate total use-solution mass may be used.

3.3. Use-solution prepared at CSF lower certified limit (LCL) – 1 active ingredient

3.3.1 Determine the active ingredient concentration (ppm) in the test substance use-solution when diluted (per label or protocol) using the test substance (concentrate) with active ingredient(s) at the LCL.

Example: 1 oz/gallon

$$\%\ \text{Dilution} = \left(\frac{1\ \text{oz Product}}{1\ \text{gallon}}\right)\left(\frac{1\ \text{gallons}}{128\ \text{oz}}\right)(100\%) = 0.781\%$$

$$\text{ppm active at LCL} = \left(\frac{\%\ \text{Active at LCL}}{100\%}\right)\left(\frac{\%\ \text{Dilution}}{100\%}\right)\left(\text{Specific Gravity} \times 10^6\right)$$

$$\text{Target mass (g) of product} = \frac{\text{ppm Active at LCL} \times \text{Total mass of use - solution} \times 100\%}{10^6 \times (\%\ \text{Active Ingredient Result})}$$

3.3.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Note: any appropriate total use-solution mass may be used.

3.4. Use-solution prepared from CSF lower certified limit (LCL) – multiple active ingredients

- Ensure that all active ingredients are at or below the calculated lower acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount (of product) that ensures all active ingredients present to be less than or equal to the calculated lower acceptance limit.

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3.4.1 Follow 3.3 to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.5. Use-solution prepared at CSF upper certified limit (UCL) – 1 active ingredient

3.5.1 Follow 3.3 and replace LCL values with UCL values.

Note: any appropriate total use-solution mass may be used.

3.6. Use-solution prepared at CSF upper certified limit (UCL) – multiple active ingredients

- Ensure that all active ingredients are at or above the calculated upper acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount that ensures any active ingredient present to be greater than or equal to the calculated upper acceptance limit.

3.6.1 Follow calculations in 3.5 (replace LCL values with UCL values) to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.7. Acceptance criteria formulas and calculations for label dilution use-solutions

3.7.1 **Example:** Product diluted at 1 oz/gallon (product/diluent)

Where: CSF UCL = 18.15%; CSF LCL = 16.43%; DF = 0.0086; Nominal (N) = 17.29%

Lower Acceptance Level = CSF LCL × DF = 16.43% × 0.0086 = 0.141%
Upper Acceptance Limit = CSF UCL × DF = 18.15% × 0.0086 = 0.156%

When the analyte of interest in the use-solution at the lower/upper acceptance limit is ≤ 1.0% after dilution; acceptance criteria may be expanded to accommodate method variability or other suitable rationale. Expanded ranges are based on 40 CFR § 158.350 (Certified Limits).

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If the nominal concentration (N) for the ingredient is	Upper/Lower Acceptance Limits after dilution may be adjusted as follows	
	Upper Limit	Lower Limit
$N \leq 1.0\%$	N + 10%	N - 10%
$1.0\% < N \leq 20.0\%$	N + 5%	N - 5%
$20.0\% < N \leq 100.0\%$	N + 3%	N - 3%

Therefore

Lower Acceptance Limit = 0.141% - 10% → [0.141% – (0.141 × 0.1)] = 0.127%
Upper Acceptance Limit = 0.156% + 10% → [0.156% + (0.156 × 0.1)] = 0.172%

Products with CSF LCL/UCL values greater than N ± 10% should follow the same range as calculated from the CSF.

Example

Lower Acceptance Limit = 0.141% - 25% → [0.141% – (0.141 × 0.25)] = 0.106%
Upper Acceptance Limit = 0.156% + 25% → [0.156% + (0.156 × 0.25)] = 0.195%

3.8. Acceptance criteria formulas and calculations for use-solutions diluted to the CSF LCL or UCL.

3.8.1 **Example:** Product diluted to 1 oz/gallon

Acceptance criteria for use-solutions diluted to the CSF LCL or UCL are greater than or equal to the Upper/Lower acceptance limits.

Acceptance Limit (Active at CSF LCL) = CSF LCL × DF = 16.43% × 0.0086 = 0.141%
Acceptance Limit (Active at CSF UCL) = CSF UCL × DF = 18.15% × 0.0086 = 0.156%

Therefore

Acceptance Criteria (Active at CSF LCL) ≤ 0.141%
Acceptance Criteria (Active at CSF UCL) ≥ 0.156%

4.0 RELATED FORMS

4.1 Form 3113: Test Substance Use-Solution Preparation for Analysis

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TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-17

5.0 REFERENCES

5.1 M032: Labeling Requirements
5.2 40 CFR 158.350

6.0 MOST RECENT REVISION SUMMARY

Revised 3.7.1.

Prepared by: Lawnida Holden Date: 20 JUL 2011
Quality Assurance: Sherri St. Clair Date: 21 Jul 2011
Management: Mary Berg Date: 21 JUL 2011

Form-Ver. 6016-06
Effective: 06/01/11
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Regulated Study Protocol Amendment

Study Title:	Aqualogic Non-Food Contact Surface Sanitizing Efficacy 30 seconds exposure time
Study Number:	1200053
Amendment Number:	1200053-1A
Amendment Effective:	May 22, 2012

Description of Amendment

The following is amended into the Pesticide Efficacy Test Section of the protocol:

Statement of Proposed Statistical Method

None

Scientific Basis for Amendment

The proposed statistical method was missing from the protocol.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

☒ *Study Sponsor* ☐ *Divisional Representative* — *Date* 05.22.2012

☒ *Study Director* ☐ *Study Monitor* — *Date* 22May2012

Printed & Verified Initial & Date MH 5/22/12

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Form-Ver. 6016-06
Effective: 06/01/11
Form Page 1 of 1

Regulated Study Protocol Amendment

Study Title:	**Aqualogic Non-Food Contact Surface Sanitizing Efficacy 30 seconds Exposure Time**
Study Number:	1200053
Amendment Number:	1200053-2A
Amendment Effective:	June 26, 2012

Description of Amendment

The protocol is amended to:

- Change the exposure time to 1 minute in the **Exposure Time/Temperature** section.
- Amend the study title to **Aqualogic Non-Food Contact Surface Sanitizing Efficacy 1 minute Exposure Time**
- Change the first sentence of the **Test Method Requirement and Test System Justification** section to read: The following apply when determining the effectiveness of a non-food contact sanitizer; 5 carriers are required on each of three samples, representing different batches one of which is greater than 60 days old.
- Aqualogic batch number 052912DT is amended into the protocol.

Scientific Basis for Amendment

- The protocol was amended to increase the exposure time to 1 minute.
- Since the exposure time was amended to 1 minute, the Study Title was amended to reflect the change in exposure time.
- The **Test Method Requirement and Test System Justification** section was amended to correct a typographical error.
- At the time of protocol initiation the batch number for this batch was unknown. The protocol was amended to identify a third batch of Aqualogic for study 1200053

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

Brent L Carter
☒ Study Sponsor ☐ Divisional Representative
7/3/12
Date

Lynn Dellickson
☒ Study Director ☐ Study Monitor
6/26/12
Date

Printed & Verified Initial & Date LMH 6/26/12

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Form-Ver. 6016-06
Effective: 06/01/11
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Regulated Study Protocol Amendment

Study Title:	**Aqualogic Non-Food Contact Surface Sanitizing Efficacy 1 minute Exposure Time**
Study Number:	1200053
Amendment Number:	1200053-3A
Amendment Effective:	August 17, 2012

Description of Amendment

The first paragraph of section B of the **CHEMICAL QUALITY VERIFICATION** portion of the protocol is amended as follows:

- The reference of the test substance batch active ingredient tested in ECOLAB GLP study number 1200052 is removed. The first paragraph of this section shall read:

> Chemical analysis will be performed on each test substance batch to determine the concentration of the active ingredient. Chemical analysis will also be performed on the test substance use-solution. The use-solution preparation will be documented in the raw data.

Scientific Basis for Amendment

The protocol was amended to allow for the testing of the test substance batch under study 1200054.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☒ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment 1200053-4A for details.
Initial & Date LAH 16Oct2012

☒ *Study Sponsor* ☐ *Divisional Representative* — *Date* 8/22/12

☒ *Study Director* ☐ *Study Monitor* — *Date* 17 Aug 2012

Printed & Verified Initial & Date LAH 17Aug2012

Form-Ver. 6016-07
Effective: 09/14/12
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Regulated Study Protocol Amendment

Study Title:	**Aqualogic Non-Food Contact Surface Sanitizing Efficacy 1 minute Exposure Time**
Study Number:	1200053
Amendment Number:	1200053-4A
Amendment Effective Date:	October 16, 2012

Description of Amendment

The protocol amendment 1200053-3A is amended to change the study number listed in the Scientific Basis for Amendment section to 1200053.

Scientific Basis for Amendment

This amendment corrects a typographical error in protocol amendment 1200053-3A.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

Brandon Cook
☒ *Study Sponsor* ☐ *Divisional Representative*
Date 10/23/2012

Lynn Dellickson
☒ *Study Director* ☐ *Study Monitor*
Date 16 Oct 2012

Printed & Verified Initial & Date LMH 16 Oct 2012

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Form-Ver. 6016-07
Effective: 09/14/12
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Regulated Study Protocol Amendment

Study Title: Aqualogic Non-Food Contact Surface Sanitizing Efficacy 1 Minute Exposure Time
Study Number: 1200053
Amendment Number: 1200053-5A
Amendment Effective Date: October 18, 2012

Description of Amendment

- The Chemical Quality Verfication section of the protocol is amended to list the CSF upper certified limit as 0.1031% free available chlorine as listed in the table below.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Free Available Chlorine*	0.0660%	0.1031%

*The equivalent weight of NaOCL (sodium hypochlorite) to the equivalent weight of Cl_2 (Chlorine) is 37.2/35.5 = 1.05. Dividing the sodium hypochlorite concentration by the ratio of the equivalent weight of sodium hypochlorite to the equivalent weight of chlorine results in the free available chlorine concentration.

- The Test Substance Concentration section of the protocol is amended to list the CSF upper certified limit as 0.1031% available chlorine as listed in the table below.

Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Available Chlorine	0.0660%	0.1031%

Scientific Basis for Amendment

The protocol was amended to update the upper certified limit to match a revised CSF. The previous value had a rounding error in the available chlorine value for the upper limit.

☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☐ This protocol amendment has been clarified and/or changed.
Refer to protocol amendment _____ for details.
Initial & Date _____

Brandon Carter
☒ Study Sponsor ☐ Divisional Representative
10/23/2012
Date

Lynne Dellinger
☒ Study Director ☐ Study Monitor
10/18/12
Date

Printed & Verified Initial & Date LAH 10/18/12

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